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Fertilizability of Coelomic Eggs in *Xenopus laevis*

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The acquisition of fertilizability in coelomic eggs of *Xenopus laevis* has been shown to be correlated with the physical, biochemical, and ultrastructural alterations of the egg envelope [coelomic envelope (CE)] induced during the passage of eggs through the pars recta portion of the oviduct. However, no direct evidence that the pars recta renders eggs fertilizable has yet been presented. In this study, we show that coelomic eggs are highly fertilizable when they are incubated with continuous shaking for 4 h at 15°C in pars recta extract (PRE) derived from females prestimulated by pregnant mare serum gonadotropin. The PRE from pituitary-stimulated *Bufo japonicus* was as potent as homologous PRE in rendering *Xenopus* eggs fertilizable. Incubation of coelomic eggs in PRE for 30 min induced a dramatic increase in the rates of sperm binding to the envelope to a level equivalent to that exhibited by the envelope from uterine eggs (VEs). The CE-to-VE ultrastructural conversion and a 43k-to-41k hydrolysis of the envelope glycoprotein component started 5 min after, and were completed by 15 min after, the start of incubation in PRE and were accompanied by an exposure of a new N-terminal sequence typical to gp41. Thus, the biochemical and ultrastructural conversions and the sperm-binding activity of the envelope induced by PREs, although being prerequisite, were not sufficient to render coelomic eggs fully accessible to fertilizing sperm.

Key Words: **oviductal pars recta; envelope conversion; oviductin; sperm binding; fertilizability; *Xenopus laevis*.**

INTRODUCTION

Eggs collected from the coelomic cavity (coelomic eggs) of amphibians are not fertilizable, but they become fertilizable after passage through the oviduct. This acquisition of fertilizability has been ascribed to secretions from two anatomically and functionally distinct portions of the oviduct, viz.: the anterior-most portion, the pars recta; and the more posterior, jelly-secreting portion, the pars convoluta (reviewed by Katagiri, 1987; Hedrick and Nishihara, 1991). The pars recta (PR) has attracted special attention because the egg envelopes become penetrable by sperm as the eggs pass through this short portion of oviduct (Grey *et al.*, 1977). In *Xenopus laevis* this change in the egg envelopes, termed the "conversion from a coelomic envelope (CE) to a vitelline envelope (VE)," has been well correlated with several alterations in the envelope, including ultrastructure (Grey *et al.*, 1977), thermostability and solubility to chemical agents (Bakos *et al.*, 1990), and hydrolytic cleavage of a specific glycoprotein component, from gp43 in CEs to gp41 in VEs (Gerton and Hedrick, 1986; Hardy and Hedrick, 1992). The PR-induced hydrolysis of the egg envelope is of particular interest because the specific target glycoprotein gp43 has been found to be a frog homologue of mammalian ZP3 (Kubo *et al.*, 1997; Yang and Hedrick, 1997). A more recent study further revealed that the CE-to-VE conversion involves the acquisition of sperm binding in the VE (Tian *et al.*, 1997b), as was also found in *Bufo japonicus* (Omata and Katagiri, 1996).

Despite these findings that indicate the apparent involvement of the oviductal PR in CE-to-VE conversion, no experiments have been performed on *X. laevis* in order to

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demonstrate directly that the secretions or extracts from the PR render coelomic eggs fertilizable, as has been demonstrated for *Bufo arenarum* (Miceli *et al.*, 1978) and *B. japonicus* (Katagiri *et al.*, 1982). Most recently, Lindsay and Hedrick (1998) reported that treatment of coelomic eggs with commercially available trypsin renders them fertilizable.

In this paper we show that coelomic *X. laevis* eggs are rendered fertilizable by incubation in PR extracts from either homologous or heterologous (*B. japonicus*) species. In addition to presenting optimal conditions for inducing egg fertilizability, we discuss the correlation between CE-to-VE conversion parameters and the acquisition of egg fertilizability upon incubation in the PR extracts.

MATERIALS AND METHODS

Materials

African clawed frogs *X. laevis* (hereafter referred to as *Xenopus*) used in this study were derived from a colony maintained in our laboratory. Toads *B. japonicus* (hereafter referred to as *Bufo*) were purchased from dealers in Tokyo during the hibernation period and were stored at 4°C until use. *Bufo* were used as a source of oviductal pars recta and jelly water but not of gametes. Thus, unless otherwise stated, the materials described in this paper refer to *Xenopus.*

Procurement of Gametes

Xenopus females were injected with 500 IU human chorionic gonadotropin "Gonatropin" (Teikoku Zoki, Co.), and eggs were obtained at 24°C from the body cavity (coelomic eggs) and ovisac (uterine eggs) 5–6 and 8–10 h after injection, respectively. Uterine eggs of *Bufo* were obtained 18 h (18°C) after the injection of homologous pituitaries.

Sperm were obtained by macerating testes in DeBoer's solution (DB: 110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, pH 7.4, by 5 mM Tris–HCl) and were stored in ice-cold DB. To inseminate the dejellied uterine eggs or jelly-less coelomic eggs, the eggs were placed in 0.5 ml of media containing jelly water in agar-based 24-well plates, and 0.1 ml of sperm suspension was added to each. The final concentrations of sperm in the insemination media were $0.5–1 \times 10^7$ cells/ml. The rates of fertilization were scored 4–8 h after insemination by counting the number of eggs undergoing cleavage.

Assay of Sperm Binding to Envelopes

To assay the binding of sperm to the VE or CE, eggs were inseminated as described above, except that the insemination medium used was always 1/3 DB. Thirty minutes after the addition of sperm, the eggs were thoroughly washed with 1/3 DB, fixed with 2.5% glutaraldehyde in cacodylate buffer, and stained with 0.3 μg/ml Hoechst 33258. The rates of sperm binding were determined by counting, under an epifluorescence microscope, the number of sperm that had bound to an area of 0.05 mm^2 of the VE or CE.

Dejellying and Preparation of Jelly Solutions

Uterine eggs from *Xenopus* were dejellied by treatment with 45 mM 2-mercaptoethanol in modified Ringer's solution (MR: 100 mM NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.8, by 5.0 mM Na–Hepes). Jelly water from *Xenopus* was prepared, according to the method of Roberts and Gerhart (cited by Heasman *et al.*, 1991), by shaking 8 ml of 1/3 MR containing 3 g of uterine eggs for 60 min. Jelly water from *Bufo* was obtained by shaking 10 ml of 1/3 DB containing 5 g of uterine eggs for 60 min. Ficoll (Sigma) or polyvinylpyrrolidone (PVP, K90; Sigma) were added to the jelly waters at concentrations of 10 and 5% (w/v), respectively. These jelly waters were referred to as JWF (jelly water containing Ficoll) and JWP (jelly water containing PVP), respectively.

Oviductal Pars Recta Extracts (PREs)

To prepare the oviductal PREs, *Xenopus* females were injected with 35 IU pregnant mare serum gonadotropin (PMSG: Serotropin, Teikoku Zoki, Co.). The frogs were kept at 24°C for 4 days after the injection, and the pars recta (PR), including both PR-1 and PR-2 portions (Yoshizaki, 1985), were dissected out, homogenized at 10 pairs PRs/ml Ca^{2+}-rich DB (DB containing 5 mM $CaCl_2$), and centrifuged at 15,000*g*. The resulting supernatant served as the *Xenopus* PREs. The *Bufo* PRE was prepared from ovulating females by homogenizing 2 pairs PRs/ml Ca^{2+}-rich DB, as reported previously (Takamune and Katagiri, 1987). Coelomic eggs from *Xenopus* were treated with PRE from *Xenopus* or *Bufo* in agar-based 24-well plates at 15°C for various periods of time, under continuous gyratory shaking at 150 rpm. After incubation in PRE, the eggs were washed with DB for further insemination or analytical studies.

Electrophoresis and Western Blotting

For electrophoretic analyses, the envelopes of eggs from the coelom (CEs) and ovisac (VEs) were collected by sieving the eggs through a nylon screen (nytal 13-100), as described previously (Takamune *et al.*, 1987). The envelopes of the PRE-treated coelomic eggs were manually peeled off under a dissecting microscope. The envelopes (0.1–0.3 envelopes/lane for Western blotting; 6 μg protein/lane for Coomassie brilliant blue staining) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970), employing 4–20% acrylamide gels (Daiichi Chemicals, Tokyo).

The mouse monoclonal antibody against the N-terminus of gp41 (designated anti-N41), which specifically recognizes hexapeptide QLPVSP, was prepared as described previously (Kubo *et al.*, 1999). For preparation of monoclonal antibodies specific to gp43 and gp41 (designated anti-gp43/41), gp41 was purified from *Xenopus* VE by SDS–PAGE followed by electroelution from the gel. Female BALB/c mice were immunized according to the protocol described by Kubo *et al.* (1999). Since preliminary experiments revealed that only gp43 and gp41 but not other envelope glycoprotein components carry the epitope of ganglioside GM2, identification of hybridomas producing anti-gp43/41 (IgG2a isotype) was carried out by the enzyme-linked immunosorbent assay (ELISA) using microtiter plates precoated with gp41 (cf. Kubo *et al.*, 1999) and with ganglioside GM2 (Kotani *et al.*, 1994) isolated from human Tay-Sachs brain as described previously (Momoi *et al.*, 1976).

The envelope glycoproteins separated by SDS–PAGE were eletroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Nippon Millipore, Tokyo, Japan) according to Towbin

TABLE 1
Jelly Substitution for Fertilization of Eggs of *Xenopus laevis*

Experiment	1/3 MR or DB	JWF[a]	10% Ficoll	JW	*Bufo* JWP[b]	Jellied eggs (control)
A	0 (23)[c]	37 (19)	10 (20)	10 (21)		30 (53)
B	0 (20)	80 (20)	86 (21)	40 (20)		44 (32)
C	0 (19)	100 (20)	64 (22)			85 (92)
D	0 (22)	100 (21)	73 (22)			100 (75)
E	0 (23)	88 (17)			96 (25)	
F	0 (22)	62 (21)			78 (36)	
G	0 (32)			30 (33)	74 (31)	
Total	0 (161)	77.8 (118)	58.3 (86)	26.7 (74)	82.7 (92)	64.8 (252)

Note. Uterine eggs were dejellied by 50 mM β-mercaptoethanol and were inseminated in the presence of various media as indicated, with sperm suspension at 5–8 $\times$ 10^6/ml (1/3 DB).
[a] Jelly water containing 10% Ficoll.
[b] Jelly water containing 5% PVP.
[c] % fertilized (no. of eggs used).

et al. (1979). The transblotted membranes were blocked with 5% skim milk in PBS and treated with the culture supernatant and peroxidase-conjugated goat anti-mouse Ig (Amersham) containing 1% skim milk in PBS. The reaction was visualized by a chemiluminescent detecting system (Amersham) according to the manufacturer's protocol.

Determination of Protein Concentrations

Protein concentrations were determined by bicinchoninic acid (BCA) protein assay reagents (Pierce), using bovine serum albumin as a standard.

Electron Microscopy

The coelomic eggs treated with PRE for various periods of time were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide, dehydrated through an ethanol series, and embedded in Epon 812. Ultrathin sections of CE, stained by uranyl acetate and lead citrate, were viewed with a JEOL JEM-100X electron microscope.

RESULTS

Acquisition of Fertilizability in Coelomic Eggs by Incubation in Pars Recta Extracts

When dejellied uterine eggs from *Xenopus* were inseminated in the presence of homologous JWF, a high rate of fertilization was obtained (Table 1). We found that JWP derived from *Bufo* was as effective as *Xenopus* JWF in supporting fertilization of dejellied *Xenopus* eggs. JWF derived from *Bufo* was also effective in supporting fertilization (not shown). Jelly water, Ficoll, or PVP (not shown) alone tended to be less effective, but together, jelly water and these synthetic chemicals synergistically acted to substitute jelly envelopes for supporting fertilization. Thus, in some experiments for the study of coelomic egg fertilization described below, we used *Bufo* JWF or JWP as a jelly substitution medium.

Coelomic eggs were placed in the PREs derived from females that had or had not been primed with PMSG, incubated for 4 h with constant shaking at 15°C, and inseminated in the presence of *Xenopus* JWF. The results presented in Table 2 clearly show that priming the females with PMSG for 4 days prior to extraction of the PRE dramatically increased the ability of the extracts to make the coelomic eggs fertilizable.

To determine the optimal conditions for incubation in PRE from PMSG-primed females, the eggs were incubated with continuous shaking in PRE for various periods of time and inseminated in the presence of JWF or JWP. The results indicated that incubation for at least 4 h is necessary to make coelomic eggs fertilizable (Table 3). Additional tests showed that even with incubation for 4 h, continuous shaking was necessary to obtain good fertilizability (data not shown).

We also found that *Bufo* PRE is as effective as *Xenopus*

TABLE 2
Effect of Priming with Pregnant Mare Serum Gonadotropin on the Activity of Pars Recta Extracts

	Treatment for 4 h (15°C) with		
Batch	Ca-rich DB	Nonprimed PRE	Primed PRE
A	0 (45)[a]	4.9 (41)	92.0 (57)
B	0 (56)	2.2 (45)	68.2 (44)

[a] % fertilized (no. of eggs used).

TABLE 3

Fertilizability of Eggs as a Function of Time of Incubation in Pars Recta Extract (PRE)

Inseminated in	Time of incubation (h) in PRE			
	0	2	3	4
Bufo JWF[a]	0 (52)[c]	26.4 (53)	21.0 (64)	92.0 (57)
Bufo JWP[b]	0 (61)	29.0 (69)	47.3 (55)	96.3 (54)

[a] Jelly water containing 10% Ficoll.
[b] Jelly water containing 5% PVP.
[c] % fertilized (no. of eggs used).

PRE in rendering *Xenopus* coelomic eggs fertilizable. As shown in Table 4, both PRE and jelly water from *Bufo* could perfectly substitute for those from *Xenopus* in supporting fertilization of *Xenopus* coelomic eggs.

PRE-Induced Acquisition of Sperm-Binding Activity in Relation to Envelope Conversions

Observation of the surface of dejellied uterine eggs inseminated in 1/3 DB revealed an uneven distribution of the number of bound sperm, with a tendency for apparently higher numbers of sperm to bind to the animal hemisphere of an egg. Thus, to count the number of bound sperm, we selected the animal hemisphere area in which the highest density of sperm was found in each egg. Assays of sperm binding to egg envelopes in this way confirmed a much lower rate of binding to CEs than to VEs (Table 5), as was reported recently by Tian *et al.* (1997b) and Lindsay and Hedrick (1998). We found that treatment of coelomic eggs with PREs from either *Xenopus* or *Bufo* dramatically increased the rate of sperm binding to the envelope (Table 5; Fig. 1).

The obvious relevance of sperm–egg envelope binding to the acquisition of egg fertilizability prompted us to determine the rates of sperm binding to coelomic eggs that had been incubated in PRE for various periods of time. As shown in Table 6, incubation for 30 min was sufficient to make the envelopes accessible to sperm binding at a level equivalent to that exhibited by VE. This result contrasts sharply with the 4 h of incubation in PRE required to attain a level of egg fertilizability equal to that of uterine eggs (cf. Table 3).

To determine the correlation between acquisition in sperm-binding activity and structural and/or biochemical alterations of the envelopes, coelomic eggs were incubated in PRE for various periods of time, and then the envelopes were collected for Western blot analyses. We found that the gp43-to-gp41 conversion in CEs started as early as 5 min after commencement of incubation in PRE and that it was completed within 15 min. This was demonstrated by a shift in the band exhibited by Western blotting using anti-gp43/41 (Fig. 2B) as well as by the occurrence of the epitope detectable by anti-N41 (Fig. 2C) between 5 and 15 min after commencement of incubation. Incubation of coelomic eggs in *Bufo* PRE induced exactly the same conversion in *Xenopus* envelopes (Figs. 2D and 2E).

When coelomic eggs were incubated in PRE from non-PMSG-primed females, the gp43-to-gp41 conversion of the envelope components detected by anti-gp43/41 occurred so slowly that only a portion of gp43 was degraded in 30 min, yielding smears of the products exhibiting the mobilities intermediate between gp43 and gp41. Under these conditions, there were no products reactive with anti-N41 (not shown). We conclude that the functional PRE-induced gp43-to-gp41 conversion in CEs accompanies an exposure of the new N-terminus detectable by our anti-N41 antibody.

As shown previously (cf. Hedrick and Nishihara, 1991), *Xenopus* CEs and VEs exhibit striking ultrastructural dif-

TABLE 4

Jelly Water (JW) and Pars Recta Extract (PRE) from *Bufo japonicus* Are as Effective as Those from *Xenopus laevis*

Batch	Inseminated in	Incubation (4 h, 15°C) in		
		Xenopus PRE	*Bufo* PRE	Ca-rich DB
A	*Xenopus* JWF[a]	18.4 (38)[c]	47.1 (34)	0 (42)
	Bufo JWP[b]	12.2 (49)	26.3 (38)	0 (44)
B	*Xenopus* JWF	95.7 (47)	92.1 (63)	0 (51)
	Bufo JWP	80.4 (46)	82.2 (45)	—

[a] Jelly water containing 10% Ficoll.
[b] Jelly water containing 5% PVP.
[c] % fertilized (no. of eggs used).

TABLE 5

Sperm Binding to Envelopes of *Xenopus* Eggs after Various Treatments

Eggs	Treatment	No. sperm/ 0.05 mm^2	Rate of binding (%)
Uterine	—	86.0 ± 21.3[a]	100[b]
Coelomic	—	6.2 ± 5.4	7.2
Coelomic	*Xenopus* PRE[c]	72.7 ± 16.1	84.5
Coelomic	*Bufo* PRE[c]	80.4 ± 20.7	94.7
Uterine	Activation[d]	4.1 ± 3.2	5.5

[a] Mean ± SD of six determinations.
[b] Expressed as a percentage of the number of sperm bound to a vitelline envelope.
[c] Treated with pars recta extracts from *Xenopus* or *Bufo* for 4 h at 15°C.
[d] Uterine eggs were activated in 1/20 DB by electric shock (40 V DC, 100 ms) and then dejellied by 2-mercaptoethanol.

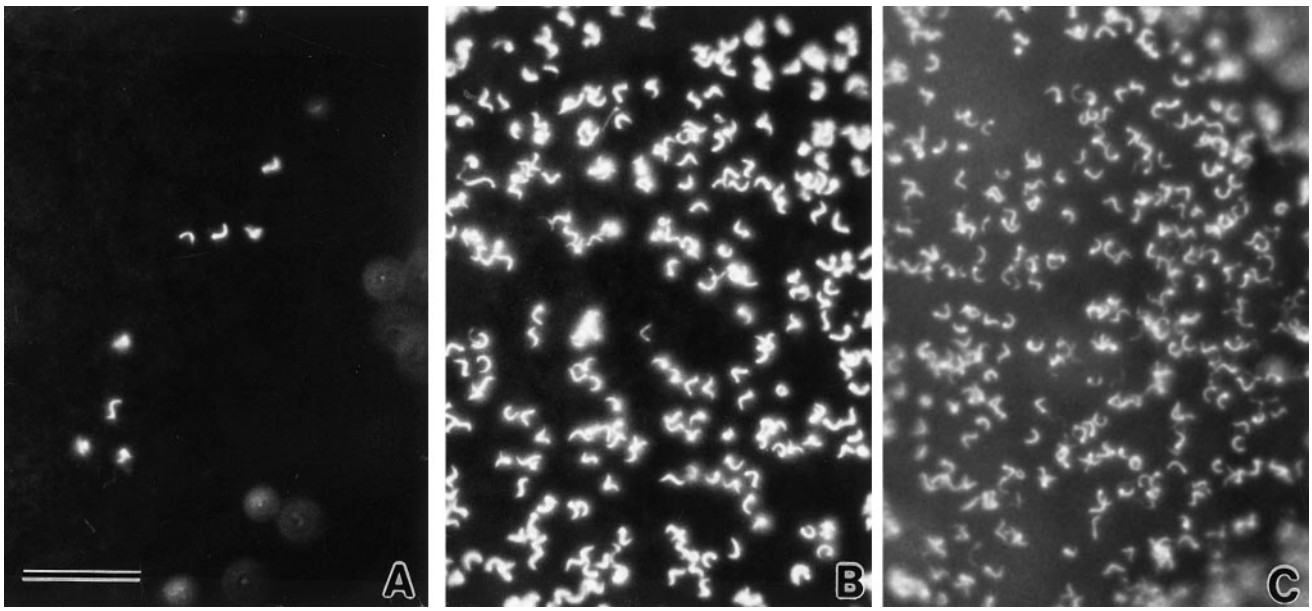

FIG. 1. Surface views of *Xenopus* eggs showing sperm binding to the envelope of a coelomic egg (A), a coelomic egg treated for 30 min with the oviductal pars recta extract (PRE) from homologous species (B), and a dejellied uterine egg (C). Note an increase of bound sperm after treatment of coelomic eggs with PRE to a level comparable to that of C. Bar indicates 50 μm.

ferences with respect to textures and distribution of constituent fibrous bundles. When coelomic eggs were incubated in PRE for 15 min, the envelope became indistinguishable from a VE with individual fibers of the fibrous bundles in the envelope becoming thinner and more evenly dispersed (Fig. 3C). This transformation was seen to have begun 5 min after the start of incubation in PRE, as demonstrated by the intermediate state of bundle dispersion shown in Fig. 3B. Thus, the CE-to-VE ultrastructural alteration coincided temporally with the gp43-to-gp41 biochemical conversion and the increase in sperm-binding activity, but not with the increase in egg fertilizability. No ultrastructural alterations were observed in the envelope when coelomic eggs were incubated in PRE from non-PMSG-primed females.

TABLE 6

Sperm Binding to Coelomic Envelopes after Treatment of Eggs with Pars Recta Extract for Various Periods

Experiment	Time treated (h)	No. of sperm/0.05 mm^2
A	0	7.5 ± 2.2[a]
	1	32.5 ± 11.7
	2	38.1 ± 10.7
	4	36.4 ± 10.1
B	0	9.2 ± 4.4
	0.5	60.8 ± 13.6
	1	51.7 ± 13.3
	3	65.7 ± 11.4

[a] Mean ± SD of six to eight determinations.

DISCUSSION

The appropriate conditions for successful fertilization of dejellied or jellyless eggs have not been studied as extensively for *Xenopus* as for other anuran species, e.g., *B. japonicus* (Katagiri, 1966), *B. arenarum* (Barbieri and Raisman, 1969), or *Rana pipiens* (Elinson, 1971). The results of this study confirmed the findings of Lindsay and Hedrick (1998) that the water-soluble components of homologous jelly with added Ficoll, originally developed by Roberts and Gerhart (cited by Heasman *et al.*, 1991), support fertilization of jellyless *Xenopus* eggs. We have also found that the jelly water from *Bufo* can substitute for *Xenopus* jelly in supporting the fertilization of *Xenopus* eggs. This may be relevant to an earlier observation that jellies from both these species, though each belonging to different families, share immunological reactivities (Hedrick and Katagiri, 1988). Although the rates of fertilization varied according to the batch of eggs and/or the jelly preparation, Ficoll or PVP tended to act synergistically with the jelly water to support fertilization (Table 1). The actual role played by each of the soluble jelly components and synthetic chemicals in these experiments is not known, but diffusible jelly components from *Xenopus* have been shown to contain a variety of fairly high-molecular-weight glycoproteins (Bonnell *et al.*, 1996).

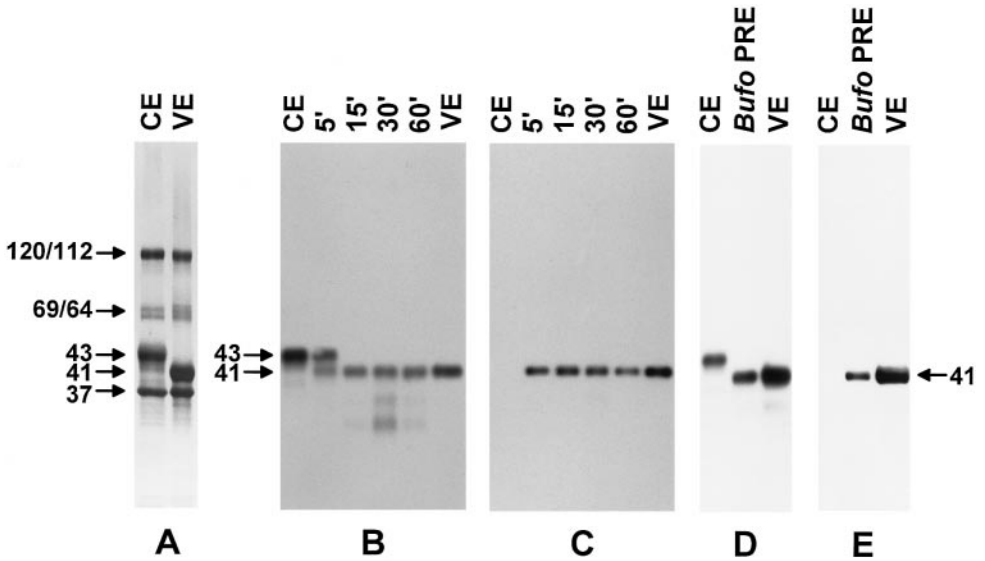

FIG. 2. (A) SDS–PAGE profiles of coelomic envelope (CE) and vitelline envelope (VE) of *Xenopus.* (B–E) Western blot analyses of envelope conversion using anti-gp43/41 (B, D) and anti-N41 (C, E), following incubation of coelomic eggs with the oviductal pars recta extract (PRE) from *Xenopus* for various periods (B, C) or with PRE from *Bufo* for 30 min. (D, E). 0–60′, given on top indicate time of incubation in minutes. Apparent molecular masses in k of envelope glycoproteins are indicated on the left and right of the membranes.

These jelly preparations may also contain heat-stable, low-molecular-weight components that evidently function as chemoattractants for sperm (Al-Azi and Chandler, 1998). The functions of the components of jelly macromolecules in supporting fertilization of *Xenopus* eggs await further systematic investigation.

The aim of this study was to directly demonstrate that the oviductal pars recta of *Xenopus* makes coelomic eggs fertilizable, as has been shown in other anurans (*B. arenarum*, Miceli *et al.*, 1978; *B. japonicus*, Katagiri *et al.*, 1982). Our study shows that, in addition to inducing changes in several chemical and physical parameters of CE-to-VE con-

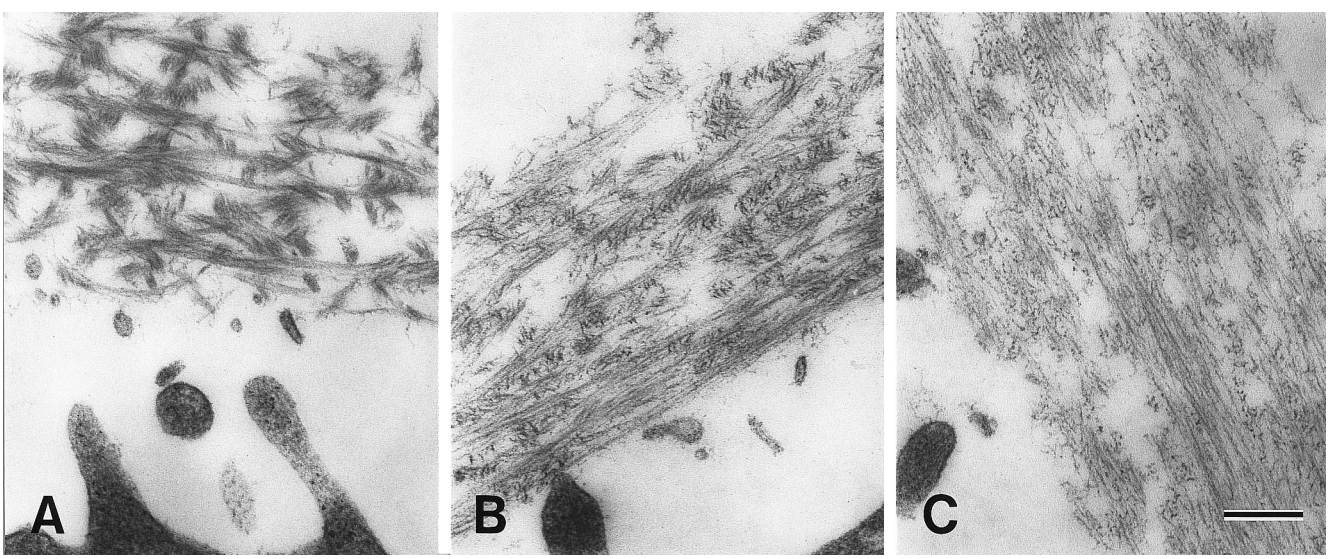

FIG. 3. TEM micrographs showing cross-sections through envelopes of a coelomic egg (A) and coelomic eggs treated for 5 min (B) and 15 min (C) with the oviductal pars recta extract. Bar indicates 300 nm.

version reported previously (Bakos *et al.,* 1990; Takamune *et al.,* 1987), the treatment of eggs with the crude PREs from both *Xenopus* and *Bufo* mimics the transition of eggs through the pars recta and induces increase in sperm–envelope binding and egg fertilizability. Thus, our results are consistent with those of recent experiments conducted in other laboratories using *Xenopus* (Tian *et al.,* 1997a,b; Lindsay and Hedrick, 1998) and with those of experiments conducted in our laboratory using *Bufo* (Omata and Katagiri, 1996), in indicating that the selective proteolysis of envelope glycoprotein by the pars recta protease affects the envelope molecular conformation so that the actual sites for binding with sperm are exposed.

The results presented here indicate, however, that the envelope conversions, as represented by gp43-to-gp41 hydrolysis, are not identical with, although they are prerequisite to, the acquisition of egg fertilizability. This is demonstrated by our finding that priming by PMSG was necessary to make PREs from *Xenopus* active in rendering eggs fertilizable (Table 2), although the secretory granules from the PR (PRGs) from nonprimed females were active enough to induce gp43 processing (Takamune *et al.,* 1987). The priming of females with PMSG reportedly gave rise to a threefold increase in proteolytic activities of PRGs (Hardy and Hedrick, 1992), while it resulted in a 30% increase of the activities in our PREs (not shown). The hormonal states of nonprimed females should be cautiously estimated because they vary according to various unspecified conditions for rearing frogs. Apparently, however, there is a lack or shortage in the PREs from nonprimed females with respect to their activities to induce ultrastructural conversion and exposure of the epitope reactive to anti-N41 in the envelope.

It remains puzzling that it takes 4 h, in contrast to 15–30 min for the envelope conversions (gp43 processing and sperm binding), to render coelomic eggs fertilizable (Table 3). The same result was also found in the PRE effects on *Bufo* eggs, where a 4-h treatment with PRE was necessary to render coelomic eggs fertilizable (Katagiri *et al.,* 1982), while a 5-min treatment was sufficient to induce the conversion of envelope components from 40–52k to 36–39k, the predicted functional *Bufo* homologue of *Xenopus* gp43 processing (Takamune *et al.,* 1986). Possible explanations for this delay in the effect of PRE are (a) successful fertilization by sperm requires the completion of other envelope alterations not monitored by us and (b) the degree of envelope conversion was not sufficient to allow fertilizing sperm to fully pass through it. Aspects of envelope alteration not monitored in this study include a PRE-induced acrosome reaction and increase in sensitivity of envelopes to "envelope (vitelline coat) lysin" of sperm, both of which have been identified in *B. japonicus* (Katagiri *et al.,* 1982) but not in *Xenopus.* The *Bufo* sperm bound to the envelopes from dejellied uterine eggs, however, have been shown not to be acrosome reacted (Omata and Katagiri, 1996). This suggests that the acrosome reaction of sperm and other related events, if also occurring in *Xenopus,* occur after sperm binding to the envelopes. Understanding exactly how sperm travel through the envelope following binding to it will explain why the PRE effects should take a long time to render the eggs fertilizable.

The most recent experiments by Lindsay and Hedrick (1998) showed that the treatment of coelomic *Xenopus* eggs with commercial trypsin fully mimicked the transit of the eggs through the oviductal pars recta, even with regard to the acquisition of egg fertilizability. This result is noteworthy in suggesting that the envelope's gp43-to-gp41 proteolysis alone is sufficient to render the eggs fertilizable. In this context, the delay in the PRE effect observed in our study may simply be due either to a low proteolytic activity of the preparations used or to a slower rate of proteolytic effect due to contamination by macromolecules in the crude extracts. The cause of this delay can be clarified by using purified oviductin to determine whether there is a correlation in kinetics between the gp43 processing, the increase in the rates of sperm binding to the envelope, and the acquisition of fertilizability.

PRGs derived from *Xenopus* and *Bufo* acted reciprocally upon each heterologous envelope to induce selective envelope glycoprotein proteolysis in exactly the same manner as induced by homologous PRGs (Takamune *et al.,* 1987). Proteases exhibiting trypsin-like characteristics, termed by Hardy and Hedrick (1992) as oviductin, have been purified from the PRGs from both *Bufo* (Takamune and Katagiri, 1987) and *Xenopus* (Hardy and Hedrick, 1991), using hydrolytic activity on arginyl peptides, such as LTR (*Bufo*) and FSR (*Xenopus*) as markers. The predicted complete amino acid sequence of *Xenopus* envelope gp43 deduced from molecular cloning (Kubo *et al.,* 1997; Yang and Hedrick, 1997) reveals, however, that there is no LTR or FSR sequence throughout the entire gp43 but that GSR sequences are present at positions 61 and 373. Based on these data, the mechanism by which oviductin generates gp41 can be proposed by a simultaneous cleavage at the C-terminus of these two GSR sequences (Kubo *et al.,* 1999). This mechanism is compatible with the acquired reactivity of gp41 to our anti-N41 monoclonal antibody that specifically recognizes QLPVSP (Fig. 2C), as well as with direct amino acid sequence data about the C-terminus of gp41 (Kubo *et al.,* 1999). Thus, to understand the exact meaning of gp43-to-gp41 envelope conversion in *Xenopus,* it is important to determine to what extent the oviductins purified from both *Xenopus* and *Bufo,* as well as the trypsin employed by Lindsay and Hedrick (1998), participate in hydrolyzing the GSR sequence and in making coelomic eggs accessible to fertilization.

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